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Evaluation of the Antimicrobial, Antioxidant, and Anti-Inflammatory Activities of Hydroxychavicol for Its Potential Use as an Oral Care Agent▽

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Hydroxychavicol isolated from the chloroform extraction of aqueous extract of *Piper betle* leaves showed inhibitory activity against oral cavity pathogens. It exhibited an inhibitory effect on all of the oral cavity pathogens tested (MICs of 62.5 to 500 μg/ml) with a minimal bactericidal concentration that was twofold greater than the inhibitory concentration. Hydroxychavicol exhibited concentration-dependent killing of *Streptococcus mutans* ATCC 25175 up to 4× MIC and also prevented the formation of water-insoluble glucan. Interestingly, hydroxychavicol exhibited an extended postantibiotic effect of 6 to 7 h and prevented the emergence of mutants of S. *mutans* ATCC 25175 and *Actinomyces viscosus* ATCC 15987 at 2× MIC. Furthermore, it also inhibited the growth of biofilms generated by *S. mutans* and *A. viscosus* and reduced the preformed biofilms by these bacteria. Increased uptake of propidium iodide by hydroxychavicol-treated cells of *S. mutans* and *A. viscosus* indicated that hydroxychavicol probably works through the disruption of the permeability barrier of microbial membrane structures. Hydroxychavicol also exhibited potent antioxidant and anti-inflammatory activities. This was evident from its concentration-dependent inhibition of lipid peroxidation and significant suppression of tumor necrosis factor alpha expression in human neutrophils. Its efficacy against adherent cells of *S. mutans* in water-insoluble glucan in the presence of sucrose suggests that hydroxychavicol would be a useful compound for the development of antibacterial agents against oral pathogens and that it has great potential for use in mouthwash for preventing and treating oral infections.

Diverse microorganisms inhabit the human oral cavity, and there is always a risk of infection with bacterial pathogens associated with the oral cavity. *Streptococcus* constitutes 60 to 90% of the remaining bacteria that colonize the teeth within the first 4 h after professional cleaning (17). Other early colonizers include *Actinomyces* spp., *Eikenella* spp., *Haemophilus* spp., *Prevotella* spp., *Propionibacterium* spp., and *Veillonella* spp. Many of the physical interactions that occur between the organisms of this community are known. *Streptococcus* is the only genus of oral cavity bacteria that demonstrates extensive and intergenic coaggregation (12, 13). The ability of this genus to bind to other early colonizers and to host oral matrices may confer an opportunity to viridians streptococci in establishing early dental plaque (17). *Streptococcus mutans* can colonize the tooth surface and initiate plaque formation by its ability to synthesize extracellular polysaccharides, mainly water-insoluble glucan from sucrose, using its glucosyltransferase (11).

The current research targeting microbial biofilm inhibition has attracted a great deal of attention, and the search for effective antimicrobial agents against these oral pathogens could lead to identification of new agents for the prevention of dental caries and periodontal diseases arising out of dental plaque formation (23). A variety of plant materials and phytochemicals, especially a class of essential oils, have long been found to exhibit effective antibacterial activity (26). The aromatic molecules derived from natural sources are being explored extensively as alternative agents in oral care products. There is some evidence that many natural molecules are good antibacterial agents that show activity against oral pathogens like *Fusobacterium nucleatum*, *Actinomyces viscosus*, *S. mutans*, *Prevotella intermedia*, *Haemophilus actinomycetemcomitans*, *Streptococcus sanguis*, and *Prophyromonas gingivalis* (3, 4, 14, 21).

Hydroxychavicol is a major phenolic compound present in the aqueous extract of the *Piper betle* leaf, which is extensively consumed as betel quid in the Indian subcontinent. The compound is better known for its antioxidant and anticancer properties (1, 9). In this study, purified hydroxychavicol from the leaves of *P. betle* was evaluated in vitro against a selected group of oral cavity pathogens especially for its effect on biofilm-forming S. *mutans* ATCC 25175 (cariogenic bacteria) and *A. viscosus* ATCC 15987 (noncariogenic bacteria).

MATERIALS AND METHODS

Extraction and isolation of hydroxychavicol from the leaves of *P. betle*. Freshly procured leaves of *P. betle* (1 kg) were extracted in boiling water (3 liters) with stirring for 4 h. The resulting extract was filtered through muslin cloth, centrifuged, and concentrated to one-sixth of the original volume under reduced pressure at a temperature of 50 ± 5°C on a film evaporator. This concentrated

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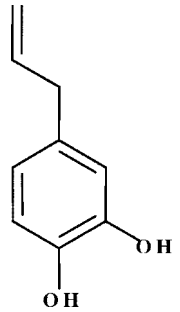


FIG. 1. Structure of hydroxychavicol.

extract was then extracted with chloroform in a separating funnel. The chloroform fraction was concentrated under reduced pressure to yield a residue (5.06 g) containing 80% hydroxychavicol, as monitored by high-pressure liquid chromatography (HPLC) and thin-layer chromatography.

The hydroxychavicol-enriched residue (5.0 gm) was chromatographed on a silica gel column (200 g; 100 to 200 mesh filter; 60 cm by 3.2 cm [Loba-Chemie, India]) using 1.0% methanol in chloroform (vol/vol) as eluting solvent. Fractions of 100 ml each were collected and subjected to thin-layer chromatography in $CHCl_3$–MeOH (19:1). The fractions containing pure hydroxychavicol were pooled, and the desired compound (Fig. 1) was crystallized from benzene-petroleum ether as a colorless solid (2.56 g) at mp 48°C (1). Hydroxychavicol was characterized by spectral analysis (19). The purity of this compound and its concentration in the crude as well as chloroform extracts were established by HPLC by following a newly developed protocol (Fig. 2).

HPLC protocol. The purity of hydroxychavicol and its concentration in the crude as well as chloroform extracts were established by Shimadzu's reverse-phase HPLC at 30°C using a Merck C_{18} column (5-μm pore size; 250- by 4.0-mm internal diameter) and UV detection at 280 nm. Sample was eluted at a flow rate of 1 ml/min with acetonitrile-water containing 1.5% acetic acid (8:92) for 5 min, and the acetonitrile concentration was increased in the gradient up to 20% over 60 min and held for 5 min, followed by a decrease in the acetonitrile concentration up to 8% over 70 min and held for 5 min. (Fig. 2).

Quantification. Hydroxychavicol exhibited a linear response in the concentration range of 17.5 μg/ml to 35 μg/ml, and the calibration curve was prepared by using the multipoint calibration curve method. A working solution was injected in different concentrations. An excellent calibration curve was obtained for hydroxychavicol ($r^2 = 0.998886$) determined on the basis of six levels of concentration.

Bacterial strains and culture conditions. The pathogenic bacterial strains were obtained from ATCC (American Type Culture Collection, Manassas, VA). *S. mutans* ATCC 25175, *Enterococcus faecalis* ATCC 29212, and *Enterococcus faecium* ATCC 8042 were maintained by subculturing on Trypticase soy agar (Difco Laboratories, Detroit, MI) at 37°C. Cultures of *A. viscosus* ATCC 15987, *S. sanguis* ATCC 10556, and *H. actinomycetemcomitans* ATCC 29522 were maintained on brain heart infusion (BHI) agar (Difco Laboratories) at 37°C in a 5% CO_2 atmosphere. *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 10953, and *Prevotella intermedia* ATCC 25611 were maintained on Wilkins-Chalgren agar (Difco Laboratories) in an anaerobic gas jar at 37°C.

Collection of clinical samples for isolation for clinical isolates. Saliva and plaque samples were collected from adults at least 25 years old attending the Orthodontic Dentistry Clinic at Corps Dental Hospital, Jammu, India. Plaque samples were collected with sterile swabs, and subgingival plaque samples were collected from four different sites with sterile paper points. In periodontal patients supragingival plaque was removed from the tooth surface before sampling. The samples were immediately transferred to transport media (Himedia, India). Each sample was then plated on duplicate blood agar plates (with 5% sheep blood). One set of plates was incubated in an anaerobic jar at 37°C for 3 to 5 days, and the other was incubated in 5% CO_2–air at 37°C for 2 days. The cultures were fully characterized to the species level by partial 16S rRNA gene sequencing and analyzed using the BLAST algorithm of the National Center for Biotechnology Information.

MIC and MBC determination of hydroxychavicol against oral cavity pathogens. The MIC was determined as per the guidelines of Clinical and Laboratory Standards Institute (formerly, the National Committee for Clinical Laboratory Standards) (16). All oral cavity bacteria used in this study were grown to stationary phase for 24 h at 37°C. Bacterial suspensions were prepared by suspending 24-h-grown culture in brucella broth (Difco Laboratories) (for anaerobic bacteria) and sterile normal saline (for aerobic bacteria). The turbidity of bacterial suspensions was adjusted to a McFarland standard of 0.5, which is equivalent to 1.5×10^8 CFU/ml. The twofold serial dilutions of hydroxychavicol were prepared in Muller Hinton broth (Difco laboratories) for aerobic bacteria, BHI broth for 5% CO_2 cultures, and Wilkins-Chalgren broth for anaerobic bacteria in amounts of 100 μl per well in 96-well U-bottom microtiter plates (Tarson, Mumbai, India). The above-mentioned bacterial suspension was further diluted in the respective growth medium, and a 100-μl volume of this diluted inoculum was added to each well of the plate, resulting in a final inoculum of 5×10^5 CFU/ml in the well; final concentrations of hydroxychavicol ranged from 15.6 to 4,000 μg/ml. The plates were incubated at 37°C for 24 h. The plates were read visually, and the minimum concentration of the compound showing no turbidity was recorded as the MIC. The minimum bactericidal concentration (MBC) was determined by spreading a 100-μl volume on a Trypticase soy agar plate from the wells showing no visible growth. The plates were incubated at 37°C for 24 h. The minimum concentration of compound that showed ≥99.9% reduction of the original inoculum was recorded as the MBC (8).

Time-kill studies against *S. mutans*. *S. mutans* ATCC 25175 was grown in BHI broth at 37°C for 24 h. The turbidity of the suspension was adjusted to 0.5 McFarland standard in sterile normal saline. A total of 200 μl of this suspension was used to inoculate 20 ml of BHI broth containing increasing concentrations of hydroxychavicol ranging from 125 to 1,000 μg/ml. Dimethyl sulfoxide controls were also included in the study. Suspensions were incubated at 37°C, and the number of CFU was determined on BHI agar using a serial dilution method at various time points (8).

Antimicrobial activity against adherent *S. mutans* in water-insoluble glucan. The formation of water-insoluble glucan by *S. mutans* was performed by a previously described method (10). Briefly, aliquots of 100 μl of culture of *S. mutans* ATCC 25175 (1×10^7 to 1×10^8 cells/ml) were inoculated into 10 ml of fresh BHI broth containing 2% sucrose (wt/vol) in the test tubes and incubated at 37°C for 24 h at an inclination of 30°. The fluid containing planktonic cells was

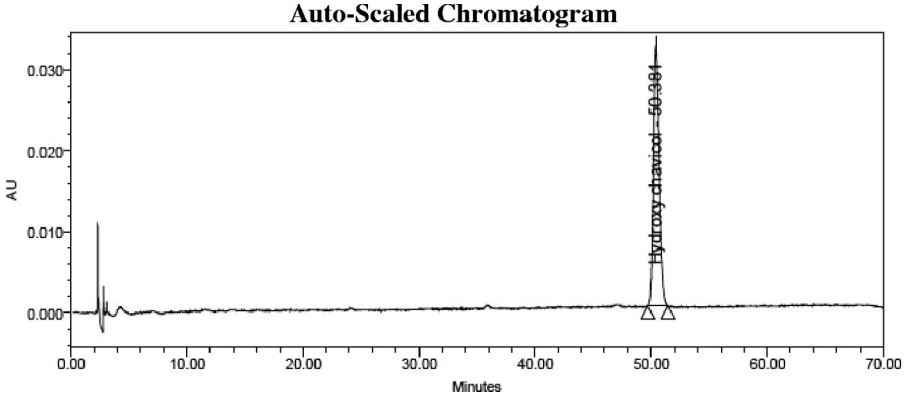


FIG. 2. HPLC chromatogram of hydroxychavicol. AU, arbitrary units.

gently removed. The water-insoluble glucan containing cells of *S. mutans* ATCC 25175 were gently washed with 10 ml of sterile water and resuspended in 10 ml of citrate buffer (10 mM, pH 6.0) containing 1,000 μg/ml hydroxychavicol, followed by incubation at 37°C for 5 min. The mixture was gently washed again with sterile water containing 0.1% Tween 80 (wt/vol), followed by the resuspension of treated cells in 10 ml of BHI broth containing 2% sucrose (wt/vol) and 0.1% Tween 80 (wt/vol). After incubation of cells at 37°C for 6, 12, 18, and 24 h, the acid produced by the culture was measured by using a pH meter. The fluid containing free cells of *S. mutans* ATCC 25175 was gently removed. The water-insoluble glucan was resuspended in 10 ml of sterile water and homogenized using five 30-s ultrasonic bursts, and the turbidity was measured at 610 nm.

PAE. The postantibiotic effect (PAE) of hydroxychavicol was determined by the method described by Crag and Gudmundsson (5). Bacterial suspensions of *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 were prepared by suspending 24-h growth in sterile normal saline. Hydroxychavicol was added at the MIC and 2× MIC into test tubes containing 10^6 CFU of each isolate per ml in BHI broth. After a brief exposure (5 min) to the hydroxychavicol, samples were diluted to 1:1,000 to effectively remove hydroxychavicol. Samples were taken every hour, and the number of CFU was determined until turbidity was noted. The PAE was calculated by the following equation: $PAE = T - C$, where T represents the time required for the count in the test culture to increase 1 $\log_{10}$ CFU/ml above the count observed immediately after drug removal and C represents the time required for the count of the untreated control tube to increase by 1 $\log_{10}$ CFU/ml.

Selection of resistant mutants in vitro. The first-step mutants of *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 were selected using a previously described method (7). A bacterial suspension containing 10^9 CFU (100 μl) was plated on BHI agar containing hydroxychavicol at concentrations equal to 2×, 4×, and 8× MIC. Mutation frequency was calculated by counting the total number of colonies appearing after 48 h of incubation at 37°C in 5% CO_2 on the hydroxychavicol-containing plate and by dividing the number by the total number of CFU plated. All mutation prevention concentration determinations were made in triplicate, and the results were identical.

Biofilm susceptibility assays. The effect of hydroxychavicol on biofilm formation by *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 was examined by the microdilution method (24). This method was similar to the MIC assay for planktonic cells. The bacterial suspensions were prepared from the overnight-grown culture, and the turbidity of the suspension was adjusted to an optical density at 610 nm (OD_{610}) of 0.7 (~1×10^9 CFU/ml). Twofold serial dilutions of hydroxychavicol were prepared in BHI broth in the wells of a 96-well flat-bottom polystyrene tissue culture plate (Tarsons, Mumbai, India) containing BHI broth in a volume of 100 μl per well. Forty microliters of fresh BHI broth was added to each well, followed by the addition of 60 μl of the above-mentioned suspension to each well of the plate. This resulted in the final inoculum of 6×10^7CFU/ml in each well; the final concentrations of hydroxychavicol ranged from 15.6 to 4,000 μg/ml. After incubation at 37°C in 5% CO_2 for 24 h, absorbance at 595 nm was recorded to assess the culture growth. The culture supernatant from each well was decanted, and planktonic cells were removed by washing the wells with phosphate-buffered saline (PBS; pH 7.2). The biofilm was fixed with methanol for 15 min and then air dried at room temperature. The wells of the dried plate were stained with 0.1% (wt/vol) crystal violet (Sigma Chemical Co., St Louis, MO) for 10 min and rinsed thoroughly with water until the negative control wells appeared colorless. Biofilm formation was quantified by the addition of 200 μl of 95% ethanol to the crystal violet-stained wells and recording the absorbance at 595 nm (A_{595}) using a microplate reader (Multiskan Spectrum; Thermo Electron, Vantaa, Finland).

The effect of hydroxychavicol was also examined on preformed biofilm. The biofilms of *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 were prepared by inoculating the wells of a polystyrene microtiter plate in a manner similar to that described above. After incubation at 37°C in 5% CO_2 for 24 h, the culture supernatant from each well was decanted, and the planktonic cells were removed by washing the wells with PBS (pH 7.2). Twofold serial dilutions of hydroxychavicol were prepared in BHI broth, and 200 μl of each dilution was added to the biofilm in the wells. The plate was further incubated at 37°C in 5% CO_2 for 24 h. The cell growth was determined by measuring the absorbance at 595 nm, and the biofilm was fixed, stained, and quantified as described above.

Propidium iodide uptake assay. The action of hydroxychavicol on cell membrane permeability of *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 cells was evaluated by the method described by Cox et al. (4). The bacterial cells were grown overnight in 100 ml of BHI broth at 37°C, washed, and resuspended in 50-mmol/liter sodium phosphate buffer, pH 7.1. The turbidity of the suspension was adjusted to and OD_{610} of 0.7 (~1×10^9 CFU/ml). A 1-ml volume of this suspension was added to a conical flask containing 19 ml of buffer and 1,000 μg/ml of hydroxychavicol. Following a 30-min incubation at room temperature, 50-μl aliquots were transferred into Eppendorf tubes containing 950 μl of phosphate buffer in fluorescence-activated cell sorting (FACS) tubes (Becton Dickinson Biosciences, CA). These tubes were stored on ice, and 5 μl of staining solution consisting of 2.5 mg/ml propidium iodide (Sigma) dissolved in MilliQ water was added to give a final propidium iodide concentration of 10 μg/ml. The cells were subjected to FACS analysis on a flow cytometer (BD-LSR; Becton Dickinson). The percentage of propidium iodide-stained cells was determined using Cell Quest Pro software (Becton Dickinson).

TABLE 1. Antimicrobial activity of hydroxychavicol against oral cavity pathogens

Organism[a]	MIC range (μg/ml)	MBC range (μg/ml)	No. of isolates
S. mutans ATCC 25175	250–500	500–1000	25
E. faecium ATCC 8042	250–500	500–1000	5
E. faecalis ATCC 29212	250–500	250–1000	5
S. sanguis ATCC 10556	125–500	125–500	16
A. viscosus ATCC 15987	250–500	500–1000	15
H. actinomycetemcomitans ATCC 29522	250–500	500–1000	5
P. intermedia ATCC 25611	125–500	125–1000	10
F. nucleatum ATCC 10953	125–250	125–500	12
P. gingivalis ATCC 33277	62.5–250	62.5–500	10

[a] All samples were from clinical isolates.

Antioxidant activity. Antioxidant activity of hydroxychavicol was measured as the inhibition of lipid peroxidation. This was measured as the content of malondialdehyde (MDA) formation induced by $FeSO_4$ plus H_2O_2 in an assay performed in rat liver microsomes (22). Microsomes (5 mg of protein/ml of 0.15 M NaCl, pH 7.0) were incubated for 20 min at 37°C in the absence (control) and presence of 100 μM $FeSO_4$ plus 50 μM H_2O_2 (stimulated). In an identical setup using Fe^{2+}-H_2O_2-stimulated incubations, 20 to 100 μg/ml hydroxychavicol in 30% dimethyl sulfoxide was added (treated). Control incubations received vehicle only. The reaction was terminated by the addition of 2.0 ml of trichloroacetic acid-thiobarbuturic acid reagent (15% trichloroacetic acid–0.375% thiobarbuturic acid [wt/vol] in 100 ml of 0.25N HCl), and lipid peroxidation content was determined as nmol of MDA formed/mg of protein.

Anti-inflammatory activity. The anti-inflammatory activity of hydroxychavicol was measured by the estimation of intracellular tumor necrosis factor alpha (TNF-α) expression in a gated population of neutrophils (2). Human blood was subjected to centrifugation at 250 × *g* for 20 min. Three layers were formed: an upper layer of platelet-rich plasma, a buffy coat middle layer, and a lower layer formed of red blood cells. The middle layer was removed and subjected to Histopaque 1077 (Sigma) gradient separation. The upper layer containing neutrophils was removed and transferred to FACS tubes (Becton Dickinson). Lipopolysaccharide (LPS) derived from *Escherichia coli* (Sigma) was added at a concentration of 10 ng/ml for the stimulation of the cells. Hydroxychavicol was added at concentrations of 2.5, 5, and 10 μg/ml. Samples were incubated for 3 h at 37°C. Controls consisted of unstimulated cells (naïve control) and LPS-stimulated cells (LPS control). Further processing was done by the addition of FACS permeabilizing solution (Becton Dickinson), followed by the addition of phycoerythrin (PE)-labeled anti-human TNF-α. (Becton Dickinson). The cells were incubated in the dark, and after being washed with sterile PBS, samples were resuspended in PBS (pH 7.4) and acquired directly on the flow cytometer (BD-LSR; Becton Dickinson). A fluorescence trigger was set on the PE (FL1) parameter of the gated neutrophil populations (10,000 events). Rolipram at 100 μg/ml was used as standard inhibitor of TNF-α in this study. Fluorescence compensation, data analysis, and data presentation were performed using Cell Quest Pro software (Becton Dickinson).

RESULTS

MIC and MBC of hydroxychavicol against oral cavity pathogens. To evaluate the antimicrobial activity of hydroxychavicol against oral microorganisms, the MICs and the MBCs were determined, and the results of 103 bacterial isolates are shown in Table 1. Hydroxychavicol exhibited an MIC range of 62.5 to 500 μg/ml against oral cavity pathogens, whereas the MBC was

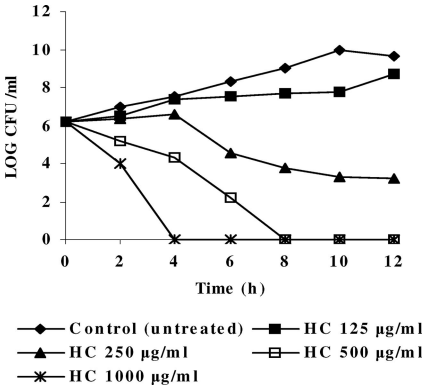


FIG. 3. Cell growth of *S. mutans* ATCC 25175 in the presence of hydroxychavicol (HC) at different concentrations.

found to be twofold greater than the inhibitory concentration, as shown in Table 1. Hydroxychavicol was equally effective on gram-negative anaerobic periodontal pathogens, such as *P. gingivalis*, and gram-positive cariogenic bacteria, such as *S. mutans*, and noncariogenic early-colonizer bacteria such as *A. viscosus*.

Time-kill studies. The time-kill kinetics studies were specifically performed against *S. mutans* ATCC 25175 owing to its importance in the initiation of plaque formation. The results of the time-kill studies are shown in Fig. 3. The MIC of hydroxychavicol (250 μg/ml) showed a 3-log reduction in growth in 10 h, compared to the untreated control, while 2× MIC and 4× MIC could reduce the CFU count of *S. mutans* ATCC 25175 below the detection limit (>50 CFU/ml) in 8 h and 4 h, respectively. The kill kinetics study showed that hydroxychavicol exhibited a time- and concentration-dependent killing effect against *S. mutans* ATCC 25175.

Activity against adherent *S. mutans* in water-insoluble glucan. The adherence of the cells to the glass surface was evident in the form of a pellicle at the interface of the liquid and glass surface when *S. mutans* ATCC 25175 was grown in BHI broth containing 2% sucrose (wt/vol) for 24 h. Changes in the OD_{610} and pH of hydroxychavicol-treated cells were compared with the untreated control (Fig. 4A B). The adherent cells of *S. mutans* ATCC 25175 treated with hydroxychavicol at 1,000 μg/ml grew slowly, and water-insoluble glucan synthesis was inhibited in the presence of sucrose. In contrast, adherent cells without hydroxychavicol treatment grew well, and there was synthesis of water-insoluble glucan in the presence of sucrose. Exposure to hydroxychavicol resulted in slower acidification of the broth in the presence of sucrose, and a pH of 6.7 was recorded after a 24-h incubation.

PAE. The PAE of hydroxychavicol was determined on *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987. Keeping in mind the potential use of hydroxychavicol as an oral care agent where the contact time of the agent is limited to a few minutes, the PAE method was slightly modified, and the bacterial cells were exposed to hydroxychavicol for 5 min only. At the hydroxychavicol MIC, the PAE was 3.5 ± 0.1 h for *S. mutans* and 4.0 ± 0.1 h for *A. viscosus*. At 2× MIC, the PAE was 6.0 ± 0.1 h and 7.0 ± 0.2 h for *S. mutans* and *A. viscosus*, respectively, which is notably long for such a brief exposure.

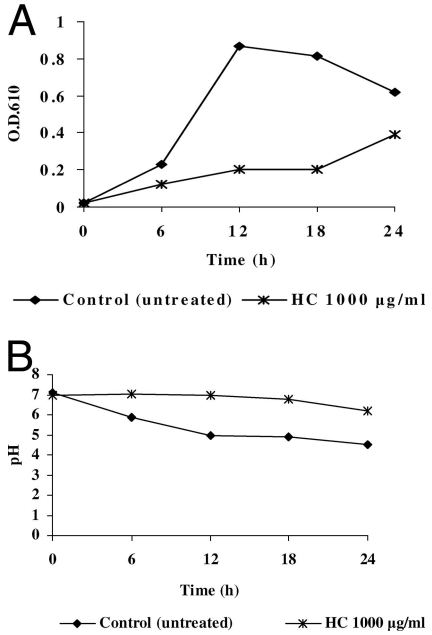


FIG. 4. Inhibitory effects of hydroxychavicol (HC) on the formation of water-insoluble glucan (A) and the drop in pH of the broth (B) in adherent cells of *S. mutans* ATCC 25175 in the presence of sucrose.

Frequency of emergence of hydroxychavicol resistance. The frequencies of mutant selection of *S. mutans* ATCC 25175 (cariogenic bacteria) and *A. viscosus* ATCC 15987 (noncariogenic bacteria) are shown in Table 2. Hydroxychavicol at 1,000 μg/ml (4× MIC) completely suppressed the emergence of mutants. This concentration of hydroxychavicol at which no mutant was selected can be defined as the mutation prevention concentration.

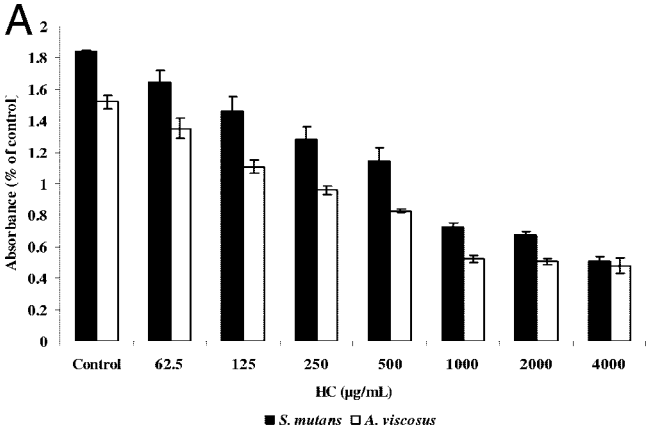
Biofilm inhibition. Hydroxychavicol exhibited an inhibitory effect on the formation of biofilm generated by *S. mutans* ATCC 25175 (cariogenic bacteria) and *A. viscosus* ATCC 15987 (noncariogenic bacteria), with a 50% minimum biofilm inhibition concentration of 500 to 1,000 μg/ml for *S. mutans* ATCC 25175 and 500 μg/ml for *A. viscosus* ATCC 15987 (Fig. 5A). Hydroxychavicol was equally effective in eradicating the preformed biofilm, with a 50% minimum biofilm reduction concentration of 500 to 1,000 μg/ml for *S. mutans* ATCC 25175 and 1,000 to 2,000 μg/ml for *A. viscosus* ATCC 15987(Fig. 5B).

Effect of hydroxychavicol on membrane integrity. Exposing the cell suspensions of *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 to a concentration of 1,000 μg/ml hydroxychavicol increased permeability to a nucleic acid stain, propidium

TABLE 2. Frequency of mutation with hydroxychavicol

Organism	Mutation frequency with hydroxychavicol at:[a]		
	2× MIC	4× MIC	8× MIC
S. mutans ATCC 25175	$<1.5 \times 10^9$	$<1.5 \times 10^9$	$<1.5 \times 10^9$
A. viscosus ATCC 19246	$<3 \times 10^9$	$<3 \times 10^9$	$<3 \times 10^9$

[a] The hydroxychavicol MIC is 250 μg/ml.



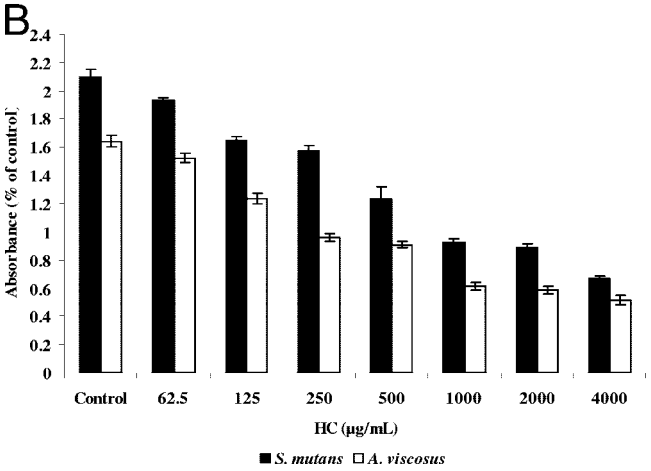


FIG. 5. Inhibitory effect of hydroxychavicol (HC) on biofilm formation by *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 (A) and reduction of preformed biofilms by *S. mutans* ATCC 25175 and *A. viscosus* ATCC 15987 (B). Values are means (± standard errors) from five independent determinations.

iodide (Fig. 6), in comparison to control suspensions that did not contain hydroxychavicol.

Antioxidant activity. Results of antioxidant activity determinations are summarized in Fig. 7. In unstimulated liver microsomes, lipid peroxidation was observed to be 1.65 nmol of

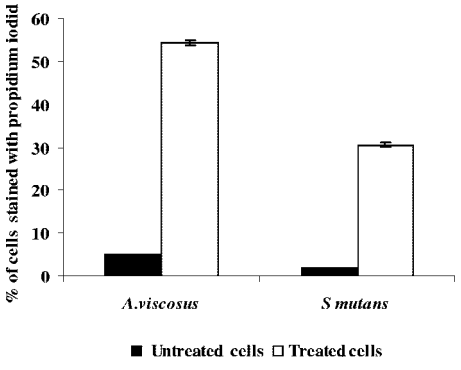


FIG. 6. Uptake of propidium iodide in cells of *A. viscosus* ATCC 15987 and *S. mutans* ATCC 25175. Cells were either untreated (control group) or treated with hydroxychavicol at 1,000 μg/ml for 15 min.

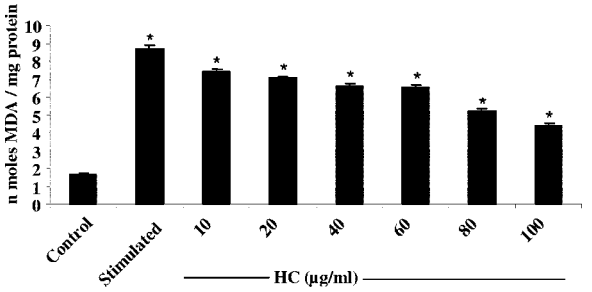


FIG. 7. Antilipid peroxidative effect of hydroxychavicol (in vitro) in rat liver microsomes. Control, basal level of MDA formation; stimulated, MDA formation in the presence of FeSO4 plus H_2O_2. Values are means (± standard errors) from six independent determinations. *, $P < 0.001$ (Student's *t* test).

MDA/mg of protein, which was increased by 5.2-fold in the presence of Fe^{2+} plus H_2O_2 (stimulated), whereas exposure of these stimulated cells to hydroxychavicol (10 to 100 μg/ml) resulted in a 15 to 50% reduction in lipid peroxidation activity.

Anti-inflammatory activity. The anti-inflammatory activity of hydroxychavicol was assessed through the estimation of the percentage of TNF-α in a gated population of neutrophils. In order to rule out the cytotoxic effects, hydroxychavicol was tested up to a maximum concentration of 10 μg/ml, which did not demonstrate any cytotoxicity in the MTT [3-(4,5-dimethylthiazol-2-yl)2 2,5-diphenyl tetrazolium bromide] assay (data not shown). In naive cells TNF-α was expressed in 1.3% of the gated population of neutrophils, which was increased twofold (approximately) in the LPS-stimulated cells. There was inhibition in TNF-α expression when these LPS-stimulated cells were exposed to hydroxychavicol at graded concentrations of 2.5, 5, and 10 μg/ml. All the tested concentrations of hydroxychavicol reduced the TNF-α expression level to below the naïve and the rolipram-inhibited control levels (Fig. 8).

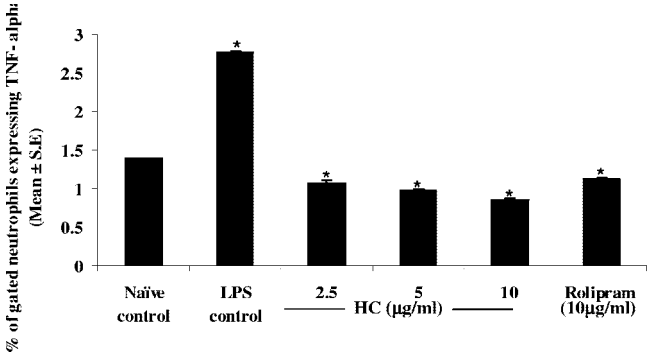


FIG. 8. Inhibitory effect of hydroxychavicol on intracellular TNF-α expression. The graph shows the percentage of TNF-α-expressing cells in the gated population of neutrophils. TNF-α-expressing cells are labeled with PE-anti-human TNF-α antibody. Naive control, basal level of TNF-α expression; LPS control, TNF-α expression after LPS stimulation. Values are means (± standard errors) from three independent determinations. *, $P < 0.001$ (Student's *t* test).

DISCUSSION

Hydroxychavicol isolated from *P. betle* was studied for its inhibitory activity against oral cavity pathogens. Hydroxychavicol demonstrated bactericidal effects against all the bacteria tested including *S. mutans*, *E. faecium*, *E. faecalis*, *S. sanguis*, *A. viscosus*, *H. actinomycetemcomitans*, *P. intermedia*, *F. nucleatum* and *P. gingivalis.* Exposure of adherent cells of *S. mutans* to hydroxychavicol effectively prevented the acidification of the suspension containing 2% sucrose (wt/vol), thus indicating the inhibition of the glucosyltransferase enzyme. Another important finding reported in this study was the long PAE exhibited by hydroxychavicol even at a brief exposure of 5 min. Hydroxychavicol at 2× MIC also prevented the emergence of mutants of *S. mutans* and *A. viscosus*.

Oral bacteria are protected by the formation of biofilms. Bacteria in a biofilm are invariably less susceptible to antimicrobial agents than their planktonic counterparts (25). Unlike the effects of hydroxychavicol on planktonic cells, as determined by the MIC and MBC, hydroxychavicol did not exhibit ≥90% reduction of the biofilm even at the highest concentration. However, in terms of the 50% minimum biofilm inhibition concentration or the 50% minimum biofilm reduction concentration, we found that hydroxychavicol not only exhibited an inhibitory effect on the formation of biofilms by *S. mutans* and *A. viscosus* but also reduced the preformed biofilm by these pathogens. Hydroxychavicol is one of the major constituents of *P. betle*, which is extensively consumed as betel quid in the Indian subcontinent. The first report of preliminary antibacterial activity of *P. betle* and hydroxychavicol was from Ramji et al. (20). This paper did not report the MICs of hydroxychavicol; however, 0.05% methanol extract of *P. betle* showed 71% and 86% inhibition in the plate dilution and broth dilution assays, respectively. Also, 0.5% hydroxychavicol inhibited the biofilm produced by anaerobes and biofilm produced in pooled saliva. Transmission electron microscopy findings by Nalina and Rahim revealed that exposure of the crude extract of *P. betle* containing 39.31% hydroxychavicol on *S. mutans* resulted in the disintegration of the plasma cell membrane (15). The increased uptake of propidium iodide in the hydroxychavicol-treated cells of *S. mutans* and *A. viscosus* in our study further confirmed the earlier findings that hydroxychavicol altered the cell membrane structure, resulting in the disruption of the permeability barrier of microbial membrane structures. Hydroxychavicol showed potent anti-inflammatory activity by significantly inhibiting the expression of the proinflammatory cytokine TNF-α. Additionally, we found that hydroxychavicol showed significant antioxidant activity, measured in terms of the inhibition of lipid peroxidation.

Hydroxychavicol is reported to have antioxidant activity (1, 6). However, like any other phenolic antioxidant, hydroxychavicol may exhibit pro-oxidant properties at higher concentrations (>0.1 mM) through the production of reactive oxygen species (1).

The natural phenols such as thymol and carvacrol are used in oral care products such as toothpaste and mouth rinse. These phenols are associated with a strong aftertaste and tingling sensations, whereas hydroxychavicol up to 2,500 μg/ml, when tasted by human subjects in our study, did not exhibit a strong aftertaste or tingling sensations (data not shown). Further, the cytotoxicity profile of hydroxychavicol was comparable to that of thymol (data not shown).

The findings reported in this study therefore strongly suggest the use of hydroxychavicol as an oral care agent. At a concentration of 1,000 μg/ml (4× MIC), hydroxychavicol can be incorporated into rinse formulation, whereas in toothpaste formulation its concentration may go up to 2,000 to 2,500 μg/ml, taking into account the dilution of the toothpaste with saliva during the process of brushing.

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